BASIC
LABORATORY PROTOCOLS

FOR THE PRESUMPTIVE
IDENTIFICATION OF

Brucella species

CDC

Centers for Disease Control
and Prevention

This protocol is designed to provide laboratories
with techniques to identify microorganisms, in order to
support clinicians in their diagnosis of potential diseases.

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I. Introduction

Brucellosis is a zoonotic disease (a disease of animals transmissable to humans) caused by infection with any of 4 species of *Brucella. B. abortus, B. melitensis,* and *B. suis* are the most common pathogenic species, with rare cases reported due to infection with *B. canis*. Although this disease is highly endemic in many countries (Peru, Mexico, Spain, Greece, Iraq, Iran, Jordan, and Kuwait), it is rare in the U.S., with approximately 100 cases per year.

The most common mode of brucellosis transmission is the consumption of unpasteurized dairy products. Brucellosis is also transmitted through direct skin contact and is an occupational hazard for farmers, veterinarians, butchers, and laboratory workers. Person-to-person transmission is not epidemiologically significant. Aerosols containing *Brucella* are considered to be highly infectious. It is estimated that inhalation of between 10 and 100 bacteria is sufficient to cause disease in people. In 1954 *B. suis* became the first agent weaponized by the U.S. in the days of its offensive biological warfare program.

The incubation period for brucellosis is highly variable, ranging from 5 days to 2 months. Acute disease is characterized by fever (usually at night), profuse sweating, malaise, headache, and muscle pain. Back pain is frequent. If untreated, a chronic form of the disease may develop. Chronic brucellosis is characterized by chronic fatigue, depression, ocular damage, and spondylitis. Naturally occurring brucellosis has a low mortality rate (>5%), yet can be extremely incapacitating and disabling.

Identification is made by culturing *Brucella* from blood or bone marrow. Due to the fastidious nature of these organisms, cultures of suspected brucellosis patients should be incubated for a minimum of 21 days prior to discard. Current blood culture systems can detect *Brucella* incubated for as little as 5 days.

Brucellosis is treatable by antibiotics but, due to the intracellular nature of the infectious process, treatment usually requires combination therapy over a long duration. Doxycycline plus rifampin for 6 weeks is recommended for uncomplicated disease in adults. Ofloxacin plus rifampin has also been reported to be effective. For complications, such as endocarditis or meningoencephalitis, triple therapy including rifampin, a tetracycline, and an aminoglycoside has been recommended. There is no available human vaccine for brucellosis.

Brucellosis is one of the most commonly reported laboratory-acquired infections. All materials suspected of harboring *Brucella* should be handled with gloves in a biological safety cabinet. Decontamination can be achieved by wiping down contaminated surfaces with a freshly-made (less than 7 days old) 1:10 aqueous dilution of household bleach.

II. Laboratory procedures for the identification of *Brucella* species

1. General

The procedures described below function to rule out presumptively identified *Brucella* spp. specimens or isolates. Laboratory coats and gloves shall be worn when processing specimens and performing tests. Safety glasses or eye shields are recommended. Any activity that brings hands or fingers in contact with mucosal surfaces, such as eating, drinking, smoking, or applying make-up shall be prohibited. Hand washing should be done prior to leaving the laboratory.

Disclaimer. Names of vendors or manufacturers are provided as examples of suitable product sources and inclusion does not imply endorsement by the Centers for Disease Control and Prevention, the United States Public Health Service, Department of Health and Human Services or the Federal Bureau of Investigation.

1.a. Handling of samples

For safety considerations, analysis of samples for biological threat agents is performed within a certified Class II biological safety cabinet (BSC). Procedures requiring removal of items from a BSC, such as slides for microscopy, should follow published microbiological practices and precautions. When using a BSC, assure that the cabinet does not contain unnecessary items that will interfere with proper airflow and function. As for any procedure involving infectious materials, standard personal protective gear should be used, such as latex gloves and lab coats, or disposable over garments. Additional respiratory protection should also be considered with materials or analytical procedures determined to be potentially hazardous outside the BSC. Once a biological agent has been identified, modifications in handling of samples can then be considered.

2. Processing of clinical specimens

2.a. Serum

For serologic diagnosis, an acute phase specimen should be collected as soon as possible after onset of disease. A convalescent phase specimen should be collected 21-28 days after the acute specimen.

2.b. Blood or bone marrow

These are the sources from which *Brucella* spp. are most often isolated.

2.c. Spleen, liver, or abscess

Brucella spp. are occasionally isolated from these sources. Selective media can be used for isolation of *Brucella* spp. from specimens with mixed flora (see below).

3. Collection and transport

3.a. Serum

Specimens should be shipped and stored frozen. If freezing is not possible, specimens can be preserved by adding 10 ml of a 1% Merthiolate solution per 1 ml of serum.

3.b. Specimens for culture

Specimens should be inoculated into appropriate culture media within 2 h of collection. If this is not possible, specimens should be refrigerated (4-10° C) until inoculation.

4. Inoculation procedure

4.a. Blood or bone marrow

Standard blood culture systems will support the growth of *Brucella* species. All inoculation procedures and manipulation of cultures should be done with gloves in a biological safety cabinet.

4.b. Tissue and wound specimens

4.b.1. Trypticase soy agar with 5% sheep blood agar (SBA) (BD Bioscience or Remel, Inc. or equivalent)
4.b.2. MacConkey agar (BD Bioscience or Remel, Inc. or equivalent)
4.b.3. Martin Lewis agar (BD Bioscience or Remel, Inc. or equivalent)

4.c. Inoculation of media

4.c.1. Blood and bone marrow: Use standard inoculation procedures for blood culture.
4.c.2. Other: Streak plates for isolation. For tissue specimens, make an impression smear for Gram-staining after inoculation.

5. Incubation requirements

5.a. Temperature and atmosphere

All cultures should be incubated at 35-37° C. Plate cultures should be incubated in 5% CO_2. Incubator humidity should be sufficient to prevent plates from drying out with prolonged (> 7 days) incubation. Humidity may also be maintained by wrapping plates with gas permeable tape.

5.b. Length of incubation

All blood cultures should be incubated for a minimum of 21 days. Terminal subcultures should be performed on negative cultures prior to discard. Subcultures should be incubated for at least 7 days.

6. Examination procedure

Plate cultures should be read daily for at least 7 days. Blood cultures should be examined using routine procedures daily for 21 days. Blind subculturing of negative cultures should be done every 7 days.

7. Presumptive differentiation of *Brucella* species

Table 1. Presumptive differentiation of *Brucella* species from similar Gram-negative genera and species.

Test	*Brucella* spp.	*Acinetobacter* spp.	*Psychobacter phenylpyruvicus* [a]
Specimen Source	blood, bone marrow	various	various
Gram stain morphology	tiny coccobacilli	broad coccobacilli	broad coccobacilli
Oxidase	+[b]	-	+
Urea hydrolysis[c]	+	v	+
X or V factor requirement	-	-	-

[a] Formerly *Moraxella phenylpyruvica*.
[b] Oxidase: *B. abortus*, *B. melitensis*, and *B. suis* are all oxidase-positive organisms. *B. canis* isolates may be oxidase-variable.
[c] Urea hydrolysis: most *Brucella* isolates vigorously hydrolyze urea. Using the Christensen's tube test, hydrolysis can be observed in as early as 15 min incubation with *B. suis* strains and within 1 day of incubation with most strains of *B. abortus*, and *B. melitensis*.
+ Greater than or equal to 90% positive;
-, less than or equal to 10% positive;
v, variable, 11-89% positive.

Table 2. Presumptive differentiation of *Brucella* species from similar Gram-negative bacteria.

Test	*Oligella* spp.	*Haemophilus influenzae*	*Francisella tularensis*
Specimen Source	urinary tract	various	various
Gram stain morphology	tiny coccobacilli	small coccobacilli	tiny coccobacilli
Oxidase	+	v	-
Urea hydrolysis[c]	+	v	-
X or V factor requirement	-	+	-[d]

[c] Urea hydrolysis: most *Brucella* isolates vigorously hydrolyze urea. Using the Christensen's tube test, hydrolysis can be observed in as early as 15 min incubation with *B. suis* strains and within 1 day of incubation with most strains of *B. abortus*, and *B. melitensis*.
+ Greater than or equal to 90% positive;
-, less than or equal to 10% positive;
v, variable, 11-89% positive.
[d] Growth enhanced by cysteine.

8. Presumptive identification of *Brucella* species
Inoculation, incubation, and differential tests

8.a. Colony morphology on SBA

Brucella will appear as punctate colonies after 48 h of incubation. Colonies are non-pigmented and non-hemolytic. All suspicious colony types should be examined by Gram stain and urea test.

8.b. Gram stain morphology

Brucella species have a characteristic Gram stain morphology that is extremely helpful in differentiating them from other Gram-negative organisms. *Brucella* cells appear as tiny, faintly stained coccobacilli.

8.c. Oxidase test (Kovac's modification)

8.c.1. Principle

The purpose of this test is to detect in bacteria the presence of oxidase enzymes associated with the cytochrome respiratory system. The reagent is a dye that acts as an electron acceptor and changes color in the presence of oxidase enzymes.

8.c.2. Specimen

This test is performed on samples of fresh colonies from agar cultures.

8.c.3. Materials

a) 0.5% tetramethyl-p-phenylenediamine (Sigma #T7394 or equivalent). Combine 50 mg of powder with 10 ml of distilled water. Store in a brown dropper bottle. The solution is stable 1 week at 4-10° C, or indefinitely when frozen at -20° C.
b) Whatman #1 filter paper
c) Disposable plastic loop

8.c.4. Controls

a) Positive control strain: *Pseudomonas aeruginosa* ATCC 35032 or equivalent
b) Negative control strain: *E. coli* ATCC 25922 or equivalent
c) Method control: Perform the test with fresh cultures of the control strains using the same method as with unknowns. Control strains should be assayed on each day of testing.
d) Resolving out-of-control results: Check the purity and identity of the control strains and repeat the test. If the control strains are pure and correctly identified, make a new batch of reagent and repeat the test.

8.c.5. Procedure

Place 1-2 drops of oxidase reagent on a piece of Whatman #1 filter paper and, using a disposable plastic loop, mix a loopful of the organisms from a 24 h plate culture into the reagent on the paper. Observe for the development of a light to dark blue color within 10 sec of inoculation.

8.c.6. Interpretation

Development of blue color within 10 sec of inoculation indicates a positive reaction (a late reaction after 10 sec is considered weak).

Caution: some metals in bacteriological loops produce false positive reactions. Platinum-iridium wire (85 platinum:15 iridium) loops are satisfactory. If another kind of loop is used, it should be tested first by exposing it to the oxidase reagent on filter paper.

8.c.7. References for oxidase test

King EO, Tatum HW: *Actinobacillus actinomycetem comitans* and *Haemophilus aphrophilus*. J Infect Dis 1111:85-94, 1962.

Kovac, N: Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 178:703, 1956.

8.d. Urea hydrolysis test (Christensen's method)

8.d.1. Principle

This test is used to determine an organism's ability to hydrolyze urea, forming ammonia by action of the enzyme urease.

8.d.2. Specimen

Fresh colonies from agar plate cultures.

8.d.3. Materials

a) Urea Agar
Solution A: Bacto-urea agar base 29 g (Difco Products/BD Bioscience #0140 or equivalent) (composed of Bacto-peptone, 1 g; Bacto-dextrose, 1 g; sodium chloride, 5 g; monopotassium phosphate, 2 g; urea, 20 g; and Bacto-phenol red, 0.012 g); distilled water 100 ml. Dissolve completely and filter sterilize. Solution B: Bacto-agar 15 g; distilled water 900 ml. Autoclave solution B at 121° C for 15 min and cool to 50° C. Then add solution A, gently mix, and tube in 5-ml amounts into 16 x 125 mm screw-cap tubes and slant. Tubes can be stored for 6 months at 4° C.
Note: Pre-made agar slants of this medium can be obtained from commercial sources (BD Bioscience, #4321097 or equivalent).
b) Bacteriologic inoculating loop

8.d.4. Controls

a) Positive control strain: *Proteus mirabilis* ATCC 25933 or equivalent
b) Negative control strain: *E. coli* ATCC 25922 or equivalent
c) Method control: Perform the test with fresh cultures of the control strains using the same method as with unknowns. Control strains should be tested on receipt of each new batch of medium.

d) Resolving out-of-control results: Check the purity and identity of the control strains and repeat the test. If the control strains are pure and correctly identified, make a new batch of reagent and repeat the test.

8.d.5. Procedure

a) Inoculate the slant with growth from a fresh agar culture.
b) Incubate at 35° C under aerobic conditions.
c) Observe for a color change to pink within 1 h. If no color change occurs, observe again after overnight incubation and at 2 days of incubation.

8.d.6. Interpretation

A positive test is indicated by the development of a pronounced pink color in the agar.

8.d.7. Reference for urea hydrolysis test

Christensen WB. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. J Bacteriol 52:461-466, 1946.

9. Actions if a presumptive *Brucella* spp. colony is identified and suspected as a bioterrorist threat agent

9.a. Preserve original specimens pursuant to a potential criminal investigation.
9.b. Contact the local FBI, state public health laboratory, and the state public health department.
9.c. Local FBI agents will forward isolates to a state health department laboratory as is necessary. Consultation with a state health department laboratory is strongly encouraged as soon as *Brucella* spp. is suspected as a bioterrorist threat agent.

10. Listed vendors

American Type Cell Culture [ATCC], 800-638-6597
Remel, Inc. 800-255-6730
BD Bioscience [BBL], 800-675-0908

III. References

Alton GG, Jones LM, Pietz DE. Laboratory Techniques in Brucellosis. 2nd ed., Geneva World Health Organization 1975, pp. 42-44.

Christensen WB. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. J Bacteriol 52:461-466, 1946.

King EO, Tatum HW: *Actinobacillus actinomycetem comitans* and *Haemophilus aphrophilus*. J Infect Dis 1111:85-94, 1962.

Kovac's N: Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 178:703, 1956.

Weaver RE, Tatum HW, Hollis DG. The Identification of Unusual Pathogenic Gram Negative Bacteria (EO King). Preliminary Revision. Atlanta, Center for Disease Control, 1972.

Weyant, R.S., C.W. Moss, R.E. Weaver, D.G. Hollis, J.G. Jordan, E.C. Cook, and M.I. Daneshvar. 1996. Identification of Unusual Pathogenic Gram- Negative Aerobic and Facultatively Anaerobic Bacteria. Williams & Wilkins, Inc. Baltimore, MD.